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Differential *hTERT* mRNA processing between young and older glioma patients

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Abstract The amplification of *hTERT* was detected in glioma tissues, although telomerase activity was not always found within these specimens. The aim of this study was to correlate the level of *hTERT* transcription with telomerase activity in two glioma age groups. *hTERT* was significantly transcribed at similar copy numbers in both age groups. However, these mRNAs translated to telomerase in 100% of the young compared to only 25% of the older patients. While *hTERT* transcription correlated directly to telomerase protein level and activity, as well as longer telomeres in the young group, such correlations were missing in the older group.

Keywords: hTERT; Telomerase; Telomere length; Age; Glioma

1. Introduction

During cell division, telomeric repeats are consistently lost leading to a critical shortening of the telomere ensuring that a cell has limited numbers of division [1–4]. The rate of cell loss is balanced by the rate of cell renewal in normal cell cycles, however, with aging, the rate of cell loss will exceed the rate of cell renewal [5]. Telomerase reactivation enables cells that are destined to senescence to confer replicative immortality without affecting other regulatory systems [6,7]. Hence cancer cells are able to proliferate unrestricted by maintaining telomere length either by the reactivation of telomerase [8] or alternative lengthening of telomeres (ALT) [9]. The presence of ALT in glioblastoma was found to correlate with long term survival [10]. However, approximately 90% of tumour cells succeed by acquiring the expression of telomerase [11,12]. Telomerase activity was detected in the different grades of gliomas, whilst none was found within the normal brain specimens [13].

The amplification of *hTERT*, telomerase catalytic subunit, was detected in glioma cell lines and primary tumours and was suggested to correlate with the expression of telomerase directly [14]. In addition, *hTERT* mRNA was found to co-localize not only in carcinoma regions but also in benign tumour and these expression patterns are correlated with the increase severity of the histopathologic changes [15]. However, previous results from our lab detected *hTERT* mRNA in glioma tissues lacking telomerase activity [16]. The aim of this research is to evaluate the correlation between *hTERT* transcription, telomerase activity and telomerase length in two glioma age groups.

2. Materials and methods

2.1. Tissue sample

Tumour samples were obtained from glioma cancer patients, admitted to the Royal Preston Hospital, UK. Control material was obtained from patients who required resection of normal brain for purposes other than primary glioma treatment. Written consent was granted prior to the tissues being used in this investigation. Brain samples were surgically dissected and frozen immediately in liquid nitrogen prior to the assay. Tissues used in the investigation included: a recurrent anaplastic astrocytoma, an anaplastic oligoastrocytoma III, a anaplastic oligodendroglioma III, a progressive ganglioma IV, eight glioblastomas and two normal brain samples.

2.2. hTERT and GAPDH transcription

mRNA was isolated from all tissues using mRNA isolation Kit (Roche, UK). Isolated mRNA (100 ng tissue) was transcribed to cDNA using a First strand cDNA Synthesis Kit (Roche, UK) which was used as a template for qRT-PCR.

Quantitative real time PCR was carried out to determine the expression of *hTERT* (214 bp) and *GAPDH* (238 bp, glyceraldehyde-3-phosphate dehydrogenase as a control) using Fast Start DNA master^{PLUS} SYBR Green 1 (Roche, UK). The PCR primer sequences specific for all the variants of *hTERT* mRNA were 5′CGTGGTTTCTGTGTGGTGTC 3′ (sense) and 5′CCTTGTCGCCTGAGGAGTAG 3′ (antisense) and for *GAPDH* primers were 5′GAGTCAACGGATTTGGTCGT 3′ (sense) and 5′TTGATTTTGGAGGGATCTCG 3′ (antisense). After an initial denaturation at 95 °C for 10 min, the samples were subjected to 35 cycles of RT-PCR (95 °C for 10 s, annealing temperature 67 °C (*hTERT*) and 55 °C (*GAPDH*) for 15 s, and 72 °C for 15 s). PCR reactions were performed in triplicate and a negative control (no DNA) was included [16].

2.3. Telomerase activity assay (TRAPeze)

Telomerase activity was performed by Telomeric Repeat Amplification Protocol (TRAP) assay (Chemicon International, USA) according to the manufacturer's protocol. Cells (10^6) were lysed in CHAPS lysis buffer (200 μl). After incubation on ice for 30 min, the lysate was centrifuged at $12000 \times g$ for 20 min at 4 °C. The supernatant from each extract (500 ng of protein) was subjected to the TRAP assay. A preincubation at 30 °C for 30 min was carried out to allow telomerase-mediated extension of the substrate oligonucleotide before the extended products were amplified by 33 cycles of PCR (94 °C for 30 s, 59 °C for 30 s, and 72 °C for 1 min).

2.4. Telomere length assay

DNA extracted from tissue sample using FastDNA kit (Q BIOgene, UK) was subjected to the TeloTAGGG Telomere length assay (Roche) according to the manufacturer's protocol. Genomic DNA (3 μg) was

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digested with restriction enzymes that did not cut within the telomeric hexanucleotide repeats (*Hinf*1 and *Rsa1*). The digested DNA was then loaded on a 0.8% agarose gel and electrophoresed for approximately 2.5 h at 5 V/cm. The DNA was transferred, hybridized with the telomere specific digoxigenin (DIG)-labelled hybridization probe, incubated with antialkaline phosphatase before the chemiluminescence signal was detected using Molecular Imager ChemiDoc XRS System, (Biorad, UK).

2.5. Immunofluorescence

A 3 μm slice was cut from each tissue FFPE block. Tissue slides were deparaffinised and re-hydrated before antigen retrieval was performed in a microwave for 20 min. The slides were then washed with TBS plus 0.025% triton (TBS-T) for 10 min, before the blocking solution was added (10% goat serum with 1% BSA in TBS) for 2 h at room temperature. The blocking solution was discarded and monoclonal primary telomerase antibody (1:2000) (Abcam, UK) was applied to the tissues and incubated overnight at 4 °C with gentle agitation. The slides were rinsed twice in TBS-T before light sensitive FITC conjugated secondary antibody (Sigma, UK) was applied and incubated for 1 h at RT. The slides were washed three times in TBS, counter stained with VECTASHIELD® (1.5 μg/ml) mounting medium with propidium iodide (PI, Vector, USA) for 10 min to allow nuclear stain and the tissue slides were visualised. Cells were visualised and scanned using Axiovert 200M LSN 510 laser scanning confocal microscope (Carl Zeiss Ltd., UK).

2.6. Statistical analyses

Student's two-tailed *t*-test for studies on mRNA copies, protein quantification, telomerase activity and telomere length were carried out using SPSS 13.0 for Windows. Values were expressed as the means ± S.D., values of $P < 0.05$ were considered to be statistically significant.

3. Results

Human gliomas and two normal brain tissue in two age groups (20–39 and 40–70 years) (Table 1) were used to assess the transcriptional levels of *hTERT* and *GAPDH* (the house keeping gene as a reference) using qRT-PCR (Fig. 1).

In order to measure all the variants of *hTERT* mRNA, specific PCR primer sequences were used in this study. *hTERT*, the rate-limiting component for telomerase activity, was significantly transcribed in all glioma tissues and was absent in the brain control tissues ($P = 0.001$). No significant differences were observed in the transcription of *hTERT* between the different glioma age groups. Similarly, no significant differences in *GAPDH* transcription was observed between cancer and normal tissues ($P = 0.357$) (Fig. 1).

Telomerase protein levels were quantified as a percentage of telomerase positive cells per sample (a total of 250 cells were counted per sample) (Fig. 1). There was a significant difference in protein level between cancers and normal tissues ($P = 0.001$). Telomerase protein was detected in four of four young patients and in two of eight older patients. Telomerase activity was measured using the TRAPeze method. While, telomerase activity was detected in the glioma tissues that expressed telomerase protein, it was not detected in tissues lacking telomerase. As expected, there was neither hTERT expression, neither telomerase activity in the normal brain tissues (Figs. 1 and 2).

Telomere length was measured in tissues used for the study, which ranged between 11.5 and 16.3 kbp in the young cancer patients and 6.1 and 10.1 kbp in the older cancer patients. For the normal patients, the length ranged from 7.4 to 9.5 kbp. A significant difference in telomere length was observed between young and old cancer patients ($P = 0.002$), and young cancer patient verses normal patient ($P = 0.02$). However, there was no significant difference between older cancer patient and normal patient ($P = 0.817$). Moreover, there is a correlation between telomerase activity and telomere length in young glioma patients, but there was no such correlation observed in the older glioma patients. Although there was telomerase activity in one of the 67-year-old glioma patient, telomere length was similar to tissues possessing no telomerase activity. A summary of the results are presented in Table 1.

4. Discussion

Telomerase regulation can be achieved either by the transcriptional regulation of *hTER* and *hTERT* or post-transcriptional alternative splicing of *hTERT* [9]. Our results showed that there is no detectable *hTERT* mRNA in the human brain tissues, which correlated with the lack of telomerase activity. This suggests that the regulation of telomerase activity occurs at the transcriptional level of *hTERT*.

Competitive RT-PCR data indicated that most cancer cells contains 1–30 molecules of *hTERT* mRNA per cell with a relatively long half-life of 2–3 h [17].

Intriguingly, all glioma tissues used in this study regardless of their grades or invasiveness, transcribed *hTERT* at approx-

Table 1
List of tissues used and a summary of the results in ascending order of age

	Diagnosis	Age	Gender	hTERT mRNA/100 ng tissues	% Cells expressing telomerase	TRAP	Telomere length
1	Glioblastoma	23	F	298	28	+	15.1
2	Progressive ganglioglioma IV	28	F	312	46.2	+	15.8
3	Recurrent anaplastic ependymona	34	F	319	31.2	+	11.5
4	Glioblastoma	38	M	310	49.4	+	16.3
5	Glioblastoma	46	F	305	0	–	7.4
6	Anaplastic oligodendroglioma III	56	M	308	7.6	–	7.7
7	Glioblastoma	60	F	298	1	–	6.2
8	Anaplatic oligoastrocytoma III	62	F	286	1	–	7.3
9	Glioblastoma	62	M	307	0.6	–	6.1
10	Glioblastoma	63	M	300	45	+	10.1
11	Glioblastoma	64	M	311	3.2	–	7.4
12	Glioblastoma	67	F	308	28.2	+	7.4
13	Normal	38	F	1	0	–	9.5
14	Normal	66	M	1	0	–	7.4

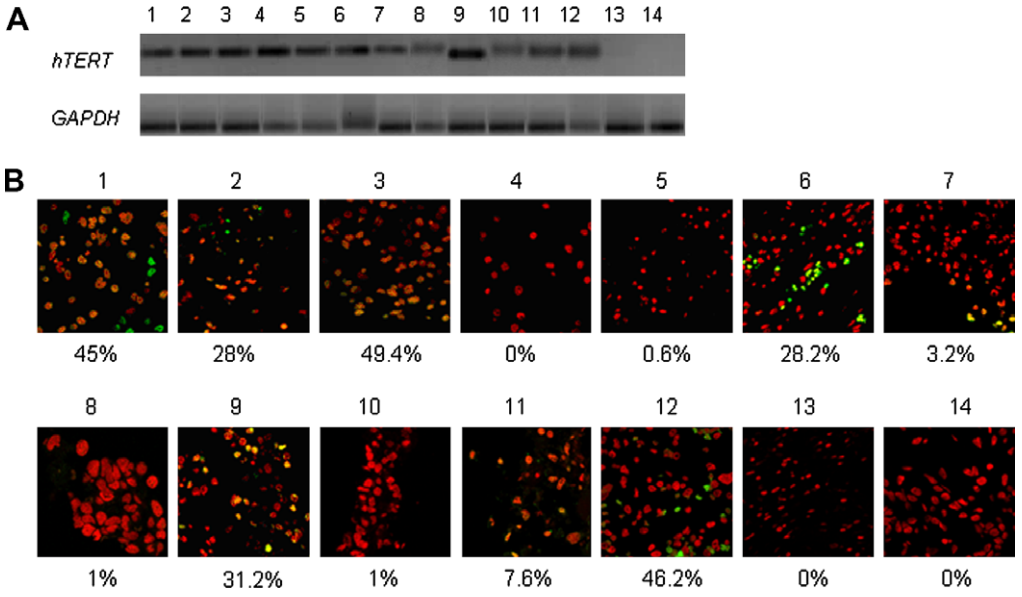

Fig. 1. Gene expression of *hTERT* and *GAPDH* together with telomerase protein levels in 14 brain tissue samples. (A) Agarose gel electrophoresis: Lanes 1–8 amplicons from glioblastoma tissues, Lane 9–12 amplicons from recurrent anaplastic ependymoma, anplastic oligoastrocytoma III, an anaplastic oligodendroglioma III, a progressive ganglioglioma IV, respectively and Lanes 13 and 14 represent the amplicons from the normal brain tissues. (B) Telomerase protein levels assessed using immunofluorescence in each corresponding tissue. Green: Telomerase antigen detected with FITC conjugate secondary antibody; red; nuclei labelled with propidium iodide. The percent represent the number of positive cells within the sample. The sample size is 500 cells (data values are mean ± S.D., n = 3).

imately similar copy numbers (300 ± 7.7 molecules per 100 ng tissues). However, the mRNA translated to telomerase in four of four (100%) of the young age compared to only two of eight (25%) of the older age tissues. This suggests that the down-regulation of telomerase protein expression in some of these tissues is not due to the lack of *hTERT* gene transcription or its promoter methylation, but possibly due to post-transcriptional or post-translational mechanisms.

A previous study showed that 32 of 33 tested renal cancer tissues expressed *hTERT* mRNA, hence only 27 of these tissues concomitantly expressed telomerase activity [18]. However, unlike our finding, *hTERT* mRNA was expressed at various levels and there was a correlation between the mRNA and telomerase activity. In addition, no correlation between telomerase activity and age was observed [18].

Variant *hTERT* mRNA splicing may produce non-functional forms; however, the extent to which *hTERT* mRNA splicing variance control telomerase activity in human cancers is controversial [17]. The distribution among the alternate splicing forms in telomerase positive cell types are strikingly similar, suggesting that the distribution among these forms is determined by common mechanisms in tumour cells that do not change significantly with tissue origin [17]. Moreover, *hTERT* mRNA splicing is not involved in the regulation of enzyme activity [19].

Two cases in this study within the older glioma age group exhibited a discrepancy between *hTERT* gene expression and telomerase activity. In addition, one case within the same age group exhibited a discrepancy between telomerase activity and telomere length. However, no such discrepancy was observed in the younger age group. Additional studies using a large cohort of glioma tissues are needed to further our understanding of the exact mechanisms for this results in the glioma older age group. Cases exhibiting *hTERT* gene transcription

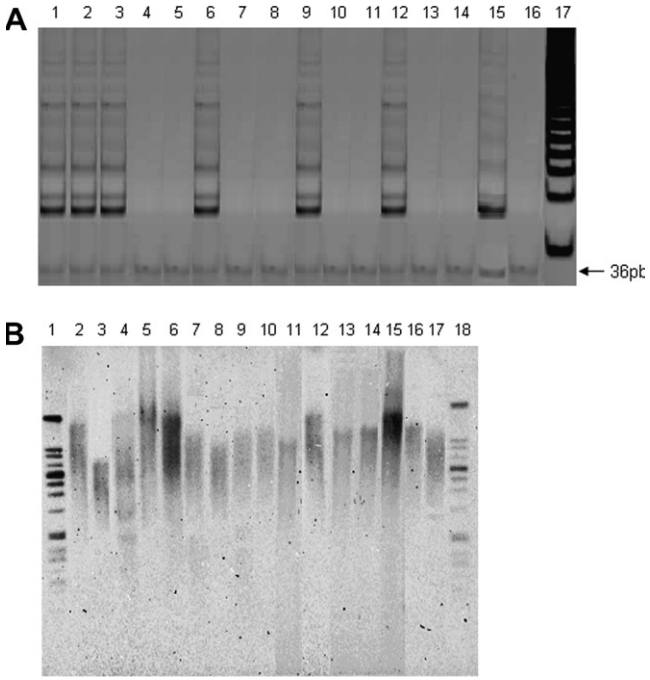

Fig. 2. Telomerase activity and telomere length in tissues. (A) Telomerase activity: Lanes 1–8 represent glioblastoma tissues, Lane 9–12 recurrent anaplastic ependymoma, anplastic oligoastrocytoma III, an anaplastic oligodendroglioma III, a progressive ganglioglioma IV, respectively and Lanes 13 and 14 represent the normal brain tissues. Lane 15 telomerase positive cells, Lane 16 CHAPS Lysis buffer negative control, Lane 17, the 20 bp molecular marker (36 bp marker as an internal control). (B) Telomere lengths: Lanes 1 and 18 represent the DIG M.Wt marker, Lane 2 DNA control high, Lane 3 DNA control low, Lanes 4–17 represent telomere length from the respective tissues as in (A).

and no telomerase activity could be explained by the fact that *hTERT* mRNA may represent an earlier event than the reactivation of telomerase enzyme in tumourigenesis [20]. Most cancer cells activate telomerase to elongate telomeres and achieve unlimited replicative potential. Interestingly, some cancer cells cannot activate telomerase and use telomere homologous recombination to ALT to elongate telomeres [9,21,22].

The hallmark question then if cells do not translate *hTERT* mRNA in certain age groups then why transcribing such a large number in cancer cell only? Could it be that these molecules can activate tumourigenesis via an alternative route in addition to the telomerase reactivation?

Evidence has suggested that telomerase contributes to tumourigenesis independent of its role in telomere maintenance [23,24].

The results suggest that additional modes of telomerase regulation act at as yet undefined levels, which may include translational and/or post-translational mechanisms and may be influenced by age. The question of whether telomerase activity is controlled at various stages of life by *hTERT* mRNA alone and/or by additional interacting proteins remains to be answered.

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